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Biochemical and Biophysical Research Communications 313 (2004) 1004–1008
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# In vitro evolution of RNA aptamers recognizing carcinogenic aromatic amines

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Received 3 December 2003

**Abstract**

The modification of cellular DNA by environmental substances is thought to be a crucial event in chemical induced carcinogenesis. Among the environmental carcinogens, aromatic amines are known for the fact that they can induce several types of cancers through the formation of so-called DNA adducts. We took advantage of the potential of the SELEX method to select for highly specific RNA ligands that recognize specific genotoxic aromatic amines. The aromatic amine 4,4′-methylenedianiline (MDA) was used as a target. Following in vitro selection, we obtained specific MDA-binding RNA molecules based on an affinity chromatography assay. These results open the possibility of using the SELEX technique to generate RNA molecules as diagnostic tools for the detection of DNA damaging compounds and ultimately DNA adducts.


*Keywords:* Chemical carcinogens; SELEX; Aptamers; DNA adducts; Affinity chromatography

Methylenedianiline (MDA) is an aromatic amine known to be an animal and suspect human carcinogen [1]. MDA is a widely used industrial chemical employed in the manufacture of plastics, azo dyes, glues, and polyurethane foams [1]. Individuals have been exposed to the compound in their workplace or because they lived in the vicinity of disposal facilities. In addition, patients on dialysis have been exposed to MDA released from polyurethane parts of the machine after sterilization [2]. MDA undergoes cytochrome P-450-mediated N-hydroxylation, followed by O-esterification to a reactive derivative capable of covalently binding to DNA or proteins [3]. Structural modifications of these macromolecules may cause alterations of the function of the genetic material. So far, biomonitoring of MDA was mainly carried out by GC/MS measurement of protein adducts [4,5] or by measuring MDA and *N*-acetyl-MDA in urine [6]. Besides, MDA is used as a biomarker of exposure to thermal degradation products of polyurethanes (e.g., 4,4′-methylenediphenyl diisocyanate (MDI)) in blood and urine of exposed workers [7,8]. However, the existing techniques of DNA damage analysis have limitations in regard to their efficiency and sensitivity or high costs involved in the analysis. Here, we propose a new approach to develop tools for the detection of aromatic amine residues, using MDA as a model. The SELEX technique [9,10] was applied to isolate highly specific RNA ligands that would recognize MDA residues. It has been shown recently that RNA aptamers selected following in vitro evolution could bind to chemically damaged DNA sites. Haller and Sarnow [11] reported on selecting aptamers that specifically recognized 7-methyl-guanosine, whereas Rink et al. [12] isolated aptamers with high affinity to 8-oxodG, a frequent oxidative lesion within DNA. But up to date, there are no reports on aptamers targeting DNA lesions caused by bulky environmental carcinogens such as aromatic amines, heterocyclic aromatic amines or polycyclic aromatic hydrocarbons. RNA aptamers fold upon

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0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.12.030

associating with their targets into three-dimensional molecular structures, where binding affinity is a result of interactions such as stacking or hydrogen bonding. Thus, the target becomes an intrinsic part of the nucleic acid structure in many reported cases [13]. The resulting aptamers have binding properties reminiscent of antibodies [14]. So far SELEX has been generally used for water-soluble targets. Here, we show for the first time that RNA aptamers can be isolated to recognize hydrophobic aromatic carcinogens.

## Materials and methods

*Oligonucleotides.* The DNA template used to generate the initial RNA pool was designed with the following oligonucleotides: 5′-GCCTGTTGTGAGCCTCCTGTCGAA$(N)_{40}$TTGAGCGTTTATTC TTGTCTCCC-3′ (DR40), 5′-*TAATACGACTCACTATA*GGGAGA CAAGAATAAACGCTCAA-3′ (F-forward), and 5′-GCCTGTTGT GAGCCTCCTGTCGAA-3′ (R-reverse), Invitrogen. DR40 contained a random region of 40 random nucleotides $(N)_{40}$ flanked by two fixed segments. F contained the promoter sequence of T7 RNA polymerase (in italics) followed by a segment complementary to the DR40 at its 3′ end and R corresponds to the 5′ terminus in DR40.

*Affinity chromatography matrix.* Epoxy-activated Sepharose 6B agarose was modified with MDA in a 30% DMF solution at pH 10 according to the manufacturer's instructions (Amersham Biosciences). Remaining active groups were blocked with 1 M Tris–HCl (pH 8). The concentration of covalently bound MDA on the column was 1.5 mM (see Fig. 1).

*Design of RNA random pool.* The three oligonucleotides F, R, and DR40 (1 μg corresponding to $\sim 2 \times 10^{13}$ DNA distinct molecules) were annealed, amplified by PCR, and purified (6% polyacrylamide gel). PCR products were used as a template for RNA synthesis in the transcription buffer (40 mM Tris–HCl, pH 8.0/5 mM $MgCl_2$/2 mM spermidine/25 mM NaCl/5 mM DTT/2 mM NTPs each/10 μCi [α-$^{32}$P]ATP (400 Ci/mmol)/0.5 U inorganic pyrophosphatase (Sigma)/175 U T7 RNA polymerase (Invitrogen)). The reaction was carried out at 37 °C overnight yielding a pool of 87-nt long RNAs (~1 nmol/100 μl). To degrade the remaining DNA template, the mixture was incubated with 10 U DNase I (Invitrogen) for 15 min. The unincorporated nucleotides were removed by G25-Sephadex chromatography (Amersham Biosciences) and the RNA pool was phenol–chloroform extracted and ethanol precipitated. Prior to affinity chromatography, RNA was denatured at 94 °C for 1 min in the binding buffer (250 mM NaCl/50 mM Tris–HCl/5 mM $MgCl_2$) and was allowed to renature for 20 min at room temperature. To suppress non-specific binding of RNA to the resin, the RNA pool was applied to a pre-column (600 μl) filled with the resin non-modified by MDA. Using a tandem-extraction set-up, the RNA pool from the pre-column in rounds 1–3 was directly eluted into the chromatographic column with the MDA-modified agarose (400 μl). After 15 min of incubation, unbound RNA was eluted with 15–20 column volumes of the binding buffer. During the selection cycles 1–4, the bound RNAs were eluted with 20 mM EDTA in binding buffer, whereas during cycles 5–7 the bound RNAs were eluted with the target itself, i.e., MDA target at 12.4 mM in the binding buffer and DMSO (99.5:0.5). At the 8th cycle, RNAs were eluted with 5 column volumes of aniline (12.4 mM the binding buffer) prior to the elution with MDA. During cycle 9 column bound RNAs were again eluted with the MDA target. After each cycle the eluted RNA was recovered by ethanol precipitation in the presence of glycogen, 20 μg/ml (Roche). Reverse transcription with the primer R was carried out using SuperScript II RNase H- Reverse Transcriptase (Invitrogen). The resulting cDNAs were subsequently amplified by PCR and the amplification products were purified by phenol/chloroform and Sephadex-G25 spin column extraction, and used as templates in the transcription step generating RNAs for the next selection cycle (see Fig. 2).

*Cloning and sequencing.* cDNA amplification PCR products were ligated into the pGEM-T Easy Vector System (Promega) in 30 mM Tris–HCl, pH 7.8/10 mM $MgCl_2$/10 mM DTT/1 mM ATP/5% polyethylene glycol/3 U T4 DNA ligase/50 ng plasmid vector and 3 ng of DNA insert and used directly to transform *Escherichia coli* DH5α competent cells (Invitrogen) by heat shock. Plasmids from individual bacterial clones were sequenced using ThermoSequenase Radiolabeled Terminator Cycle Sequencing Kit (USB, Cleveland, Ohio).

*Determination of binding constants.* DNA templates were produced by PCR amplification of individual plasmids and were subsequently transcribed to produce unique sequence RNA aptamers. Individual molecules were purified by electrophoresis on 6% polyacrylamide/8 M urea gels and eluted from the gel with a mixture of 500 mM ammonium acetate and 0.01% SDS. Dissociation constants ($K_d$) were estimated by the method of isocratic elution using the following relations: $K_d = [L_c] \times (V_c - V_n)/V_e - V_n)$, where $L_c$ denotes the ligand coupled to the matrix/$V_c$ is the column volume/$V_n$ is the void volume of the column/$V_e$ is the elution volume in the absence of free ligand and $K_d = [L] \times (V_{el} - V_n)/V_e - V_{el})$, where $L$ is the free ligand and $V_{el}$ is the elution volume for RNA in the presence of free ligand in buffer [15]. We calculate the $K_d$ of three selected different aptamers (M1, M3, and M22) to be ≈0.45, 2, and 12 μM, respectively.

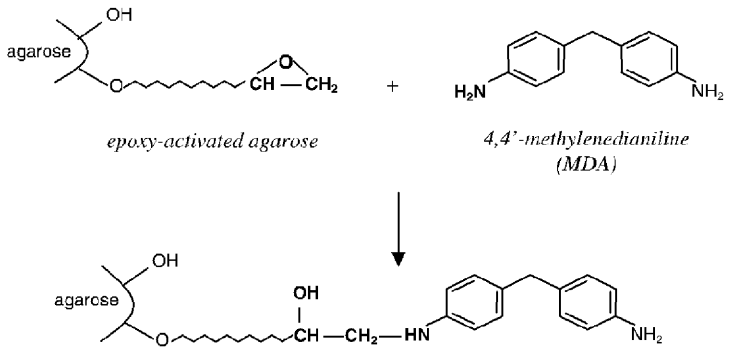


Fig. 1. Structure of the MDA-modified epoxy agarose matrix.

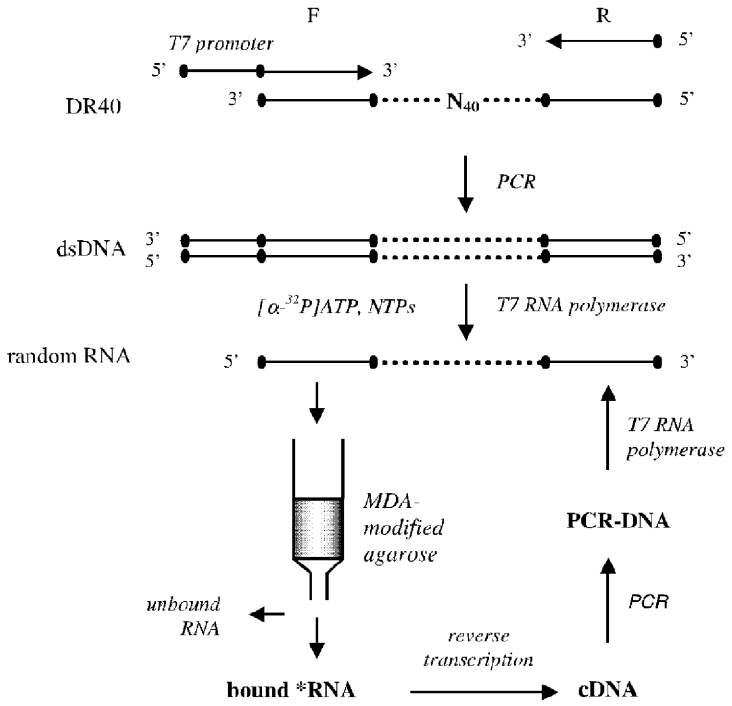


Fig. 2. Generation of RNA molecules for the in vitro evolution procedure: (F) Forward primer, containing the T7 transcriptional promoter region. (R) Reverse primer. DR40: the 87-mer ssDNA carrying the 40 nucleotide random region. DsDNA: the 104-bp template generated by PCR amplification that is transcribed into ssRNA (random RNA) in the presence of NTPs and $^{32}$P-labeled ATP. The so-generated RNA pool is subsequently applied to the MDA-affinity matrix. Unbound RNA molecules are washed off, bound molecules are collected, reverse transcribed, and PCR-amplified.

## Results

In vitro evolution experiments start with a large pool of random nucleotide sequences, followed by repeated cycles of enrichment for sequences with the desired properties and subsequent amplification of the enriched pool. Our SELEX experiment started with a pool of RNAs that were completely randomized at 40 positions, encompassed by two fixed regions that resulted in a complexity of $\sim 10^{13}$ different sequences. Multiple rounds of enrichment resulted in the exponential increase of the best binding ligands until they dominated the population of sequences. Affinity chromatography on an MDA-modified epoxy–resin was applied to select the best binding RNA molecules. Subsequent steps of reverse transcription, PCR amplification, and subsequent RNA synthesis produced the following RNA pool for selection. In total 9 cycles of selection were implemented. The retrieval of RNA molecules after each cycle of selection is demonstrated in Fig. 3. During the first 4 cycles, elution with 20 mM EDTA resulted in a slight increase of the binding RNA. Starting from the 5th cycle, aptamers were eluted with free target, which increased the yield of bound RNA species to 11% in cycle 7. The counter-SELEX procedure (see Fig. 4) did not lead to a decrease in the amount of MDA-binding RNAs, but to their further increase of up to 19%. Finally, in the 9th cycle of selection the yield of binding RNA species of 25% was obtained with free target elution. After this cycle the enriched RNA was reverse transcribed,

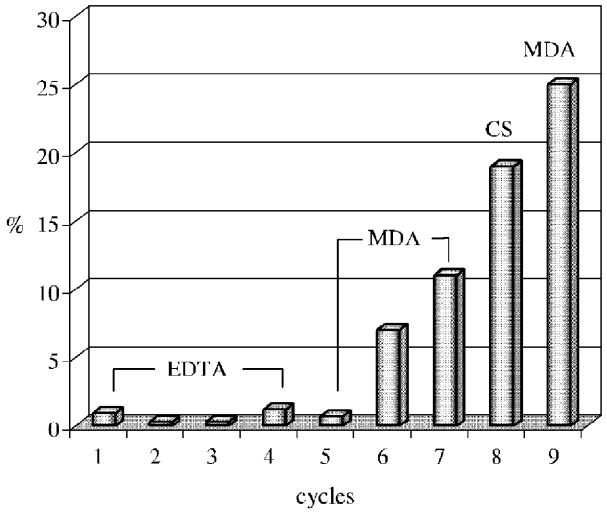


Fig. 3. Percent of RNA recovery at different cycles of selection. CS: counter selection.

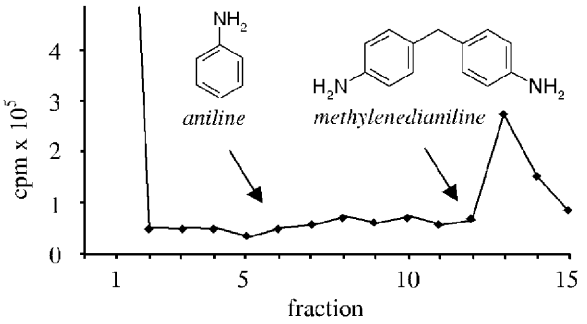


Fig. 4. Counter-SELEX: 8th cycle of selection, representing elution profile applying aniline followed by target elution (MDA).

amplified, and cloned into the pGEM-T Easy Vector System for dideoxynucleotide sequencing. Out of the 47 clones analyzed eight distinct sequences were observed (see Fig. 5). Twenty-three aptamers were represented by 18 copies of clone M1 and five copies that differed from M1 only by a single nucleotide substitution. The sequence M3 appeared eight times with one copy differing at one nucleotide position, whereas the sequence M22 occurred five times with one of the copies differing again by a single substitution. The sequences of clones M2 and M37 were observed only once, while a single nucleotide deletion distinguished two variants of clone M24 (for details see Fig. 5).

No obvious similarities in the predicted RNA secondary structure were observed between these different sequences which does not preclude that these might share certain three-dimensional folding patterns. The single nucleotide differences as between M1 and the derived clones are likely to represent errors in reverse transcription and/or DNA replication during PCR. The most representative sequences of clones M1, M3, and M22 were chosen for further characterization. The MDA-affinity-column was run under isocratic conditions to determine an approximate dissociation constant ($K_d$) between the selected RNA-ligands and the [15,16]. For the aptamers corresponding to the M1 sequence, $K_d$ of ~0.45 μM was estimated while for those corresponding to M3 a $K_d$ of ~2 μM and for those representing M22 a $K_d$ of ~12 μM were calculated.

## Discussion

We applied nine rounds of SELEX to isolate RNA aptamers specific to the chemical carcinogen methylenedianiline. After the 8th cycle we applied the counter-SELEX procedure, using aniline, which differs from MDA by the absence of a relatively bulky aromatic ring to eliminate aptamers that recognize targets similar to MDA. Highly selective aptamers have been generated towards aminoglycodide antibiotics [17] and the isoalloxazine moiety in flavine adenine dinucleotide [18]. Among others, Jenison et al. [19] and Rink et al. [12] have described the counter-SELEX procedure for molecules differing from the target only by a methyl or oxo-group. As shown in Fig. 4, elution of RNA molecules with aniline resulted in a slight increase of RNA molecules, demonstrating that after the 8th cycle the majority of RNAs could discriminate between molecules differing by an aromatic ring and hence only eluted from the column in the presence of MDA. Out of the series of aptamers identified after the 9th SELEX round, none shared a primary sequence relationship with each other, indicating that specific molecular recognition could be achieved by different RNA sequences. Likewise, there seemed to be no similarities concerning the secondary RNA structures, as analyzed with mfold (Zuker). Analyzing the binding affinities of three representatives of the most frequent sequences demonstrated that selected RNAs bind to their targets in a highly specific fashion with dissociation constants in the low micromolar range.

With the availability of aptamers binding to carcinogenic compounds, two scenarios are feasible in the future. (1) The aptamers could serve as new diagnostic tools for the detection of lesions in DNA caused by

```
          1         10        20        30        40        50
clone     |---------|---------|---------|---------|---------|    #
M1          CUGCGAJCAGGGGUAAAUUUCCGCGCAGGCUCCACGCCGC             23 (4, 1)
M2            CUCCAGUCCUCUUGAGCGGUUCCJACUUCCCUCUGCUGUG            1
M3            CAGAGGGAGUACUGAUCAACCCGAGCCUUCCGUCCCUGAG            8 (1)
M37       ACGUACGAJCCAUCCCCCUCUCGUAGUCGGGGUCACAUUCU               1
M8          CGGGUAGAGCGAGUAACUCCUGCGCCUCCAUGCGUACGGG              5
M44         CCGAGACGAGU-AGUUCCCCUUGC-CCCCUGCGCGGUCAGGG            2
M22           UGGG-AG-GAGUGCUCCUCUCCAGGCACUCUUGUUGCUCGGA          5 (1)
M24           UGGGUAG-GGGCGCUCCUCGUGAGJUUCCUCUGCUAC-GGAU          2 (1)
consensus ------cgag-ag-g----u---ucgu-cg---c-c--gcu-c---g--
```

Fig. 5. Sequence analysis of 47 RNA aptamers. Sequences shown comprise only the 40-nucleotide region that was completely randomized at the beginning of the SELEX experiment. Lowercase letters in the consensus line indicate a sequence similarity of ≥50% consensus, in the aptamer sequence nucleotides are highlighted in bold, respectively. Numbers on the right-hand side indicate how many times a specific sequence of a clone occurred, in brackets are the number of clones that differed only by a single nucleotide (position underlined), variants of clone M1: four times a G→U and one time a G→A substitution, variants of clone M3: one time a C→A substitution, variants of clone M22: and one time a C→G substitution and in the other variant of clone M24 the underlined nucleotide was deleted.

environmental genotoxins as an alternative to current detection methods. Stojanovic and Landry [20] described the development of an aptamer based colorimetric assay. Conceivably, the selection of different RNA aptamers with the SELEX technique could result in a library of different nucleic acids each capable of recognizing a specific DNA damage. (2) A second application would be the use of the selected aptamers for analytical analyses, an area that is relatively unexplored. Recently, Deng et al. [21] and Rehder and McGown [22] demonstrated that affinity chromatography columns immobilized with aptamers could selectively retain and separate nucleosides and proteins, respectively. They showed that immobilization of aptamers had little effect on aptamer-target interaction, demonstrating that it is possible to design aptamer based stationary phases able to separate compounds on the basis of the aptamer recognition. Hence, MDA specific aptamers may possibly be used in the screening for MDA in blood and urine of exposed workers, where this compound is already being used as a biomarker of exposure to the products of thermal degradation of isocyanates [7,8]. Analytical applications of these highly selective binding species could trigger new approaches for chemical separations based on differential affinity. In conclusion, SELEX provides an interesting platform for the isolation of RNA aptamers that could be used as new diagnostic tools for the identification of environmental carcinogens and in particular those that damage the DNA.

## Acknowledgments

Ulrike Brockstedt was supported by a fellowship from the DFG (Deutsche Forschungsgemeinschaft) BR 2050/1-2 and Wolfgang Pfau was supported by the Philip Morris External Research Program. The funding from the Fondation de l'Hôpital Sainte-Justine /Power Corporation Inc. is gratefully acknowledged.

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